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EFFECT OF VITAMIN D AND DIETARY CONTENT
OF CALCIUM AND PHOSPHORUS ON PROTEIN
SYNTHESIS IN RAT DUODENAL MUCOSA

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data suggest that vitamin D causes an increased synthesis of new epithelial cells in the duodenum while at the same time decreasing the time required for their maturation and sloughing.

Gel electrophoretic separation of the soluble proteins from the mucosa suggested the presence of a pre-CaBP in rats on both diets before they were treated with vitamin D. Also, the D-deficient rats fed diet 2 had a band which appeared in the same region as CaBP. When vitamin D was administered, the pre-CaBP disappeared in both groups while the CaBP was evident in both groups. Labeled amino acids did not contribute any significant radioactivity to the pre-CaBP or CaBP region indicating that the CaBP formed during the 48-hour test period did not require de novo protein synthesis.

Vitamin D had no noticeable effect on kidney protein synthesis in rats.

ABSTRACT

Forty-eight hours after the administration of vitamin D to D-deficient rats, there was less total protein in the mucosa of the duodenum than in comparable rats not treated with the vitamin. During this time there was a shift in the proportion of protein contributed by each of the fractions measured. Generally, the soluble protein fraction diminished while the nuclear debris fraction increased. For those fed diet 1 (1.2% Ca: 0.02% P), vitamin D increased the proportion of mitochondrial and microsomal protein, while those fed diet 2 (0.0% Ca: 0.3% P) showed no marked change in these particulate fractions. These data suggest that vitamin D causes an increased synthesis of new epithelial cells in the duodenum while at the same time decreasing the time required for their maturation and sloughing.

a pre-calcium binding protein

Gel electrophoretic separation of the soluble proteins from the mucosa suggested the presence of ~~1~~ (pre-CaBP) in rats on both diets before they were treated with vitamin D. Also, the D-deficient rats fed diet 2 had a band which appeared in the same region as CaBP. When vitamin D was administered, the pre-CaBP disappeared in both groups while the CaBP was evident in both groups. Labeled amino acids did not contribute any significant radioactivity to the pre-CaBP or CaBP region indicating that the CaBP formed during the 48-hour test period did not require de novo protein synthesis. ✱

Vitamin D had no noticeable effect on kidney protein synthesis in rats.

PREFACE

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INTRODUCTION

In a series of papers which appeared beginning in 1937, Nicolaysen (1-5) established that vitamin D increased absorption of calcium by the intestine. Since then an extensive volume of literature has appeared which has sought to define the mechanism(s) involved in absorption of calcium, and the role of vitamin D in relation to this process. Recent reviews (6-9) describe the history and development of our knowledge in this area. Current research seems to be directed toward understanding the factors which control three separate but interrelated steps: 1) Movement of calcium from the lumen of the intestine into epithelial cells of the mucosa; 2) Passage of calcium across the cell to the basement membrane; 3) Release of calcium from the epithelial cell across the basement membrane into the blood stream. As can be seen, the question of where and how vitamin D enhances the absorption of calcium may depend on its acting at one or more of these steps. Its mode of action remains unknown.

Recent evidence indicates that vitamin D must be converted to 1,25 dihydroxycholecalciferol ($1,25-(OH)_2D_3$) before its effect on calcium absorption may be observed. The scheme proposed by Omdahl and DeLuca (8) suggests that $1,25(OH)_2D_3$ activates a calcium dependent ATPase-alkaline phosphatase enzyme in the brush border of the epithelial cell. In concert with calcium-binding protein (CaBP), which acts as a "concentrator," calcium ions move from the lumen across the brush border into the cell. The mineral traverses the cell toward the basement membrane where it is moved out of the cell in the presence of Na^+ , depending on an exchange of ions across the membrane.

Other biochemical events must somehow be included in the proposed scheme for calcium absorption since they also appear following administration of vitamin D to a D-deficient animal. An as-yet-undefined energy-requiring process is necessary for movement of calcium from the intestinal lumen into the mucosa. This is an active process causing calcium to move against a concentration gradient (10,11). It has also

1. Nicolaysen, R., Biochem. J. 31:107, 1937.
2. Nicolaysen, R., Biochem. J. 31:122, 1937.
3. Nicolaysen, R., Biochem. J. 31:323, 1937.
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5. Nicolaysen, R. et al, Physiol. Rev. 33:424, 1953.
6. Wasserman, R. H. and Corradino, R. A., Ann. Rev. Biochem. 40:501, 1971.
7. Wasserman, R. H. and Taylor, A. N., Ann. Rev. Biochem. 41:179, 1972.
8. Omdahl, J. L. and DeLuca, H. F., Physiol. Rev. 53:327, 1973.
9. Olson, E. B. Jr., and DeLuca, H. F., World Rev. Nutr. Dietetics 17:164, 1973.
10. Schacter, D. and Rosen, S. M., Am. J. Physiol. 196:357, 1959.
11. Walling, M. W. and Rothman, S. S., Am. J. Physiol. 217:1144, 1969.

been observed that vitamin D administration stimulates incorporation of orotic acid into RNA (12) and stimulates phospholipid (13) and DNA synthesis in intestinal mucosa (14).

All the above events following the administration of vitamin D may be thought of as being due to an activation of existing enzymes or to de novo synthesis of enzymes and other proteins such as CaBP. Zull et al. (15) and Norman (16) have reported that actinomycin D (A_D) given prior to vitamin D inhibits the vitamin D effect on calcium absorption by the intestine. Since A_D is known to inhibit synthesis of mRNA from DNA, and thereby to inhibit protein synthesis, these investigations (15,16) would indicate that vitamin D manifests its function through de novo synthesis of a protein. Further support for such a concept can be found in reports by Wasserman and his colleagues (17,18) which established that administration of vitamin D to a D-deficient rat or chick caused appearance of a new protein band located in the mucosa of the small intestine which had a uniquely strong affinity for calcium. Also, MacGregor et al. (19) have shown that 20 IU of D_3 administered to D-deficient chicks will result in specific incorporation of [3H] leucine into CaBP. More recently, Lawson and Emtage (20) have demonstrated that CaBP synthesis in a cell free system is dependent on the presence of polysomes from D_3 treated chick gut. Polysomes from rachitic chick gut were inactive in the same test system.

Despite evidence accumulated in support of vitamin D-related protein synthesis, it now appears that other interpretations are possible. In 1969, Ziporin et al. (21) reported that A_D given to D-deficient rats two hours prior to vitamin D did not significantly reduce uptake of calcium by intestinal slices in vitro. An expanded version of that work was reported in 1973 (22). This would indicate that vitamin D did not require de novo synthesis of protein in intestinal mucosa in order to manifest its

-
12. Stohs, S. J. et al, Biochemistry 6:1304, 1967.
 13. Thompson, V. W. and DeLuca, H. F., J. Biol. Chem. 239:984, 1964.
 14. Birge, S. and Alpers, D. H., Clin. Res. 19:387, 1971.
 15. Zull, J. E. et al, Science 149:182, 1965.
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 17. Wasserman, R. H. and Taylor, A. N., Science 152:791, 1966.
 18. Wasserman, R. H. et al, J. Biol. Chem. 243:3978, 1968.
 19. MacGregor, R. R. et al, Biochim. Biophys. Acta 222:482, 1970.
 20. Lawson, D. E. M. and Emtage, J. S., Vitamins Hormones, 32:277, 1974.
 21. Ziporin, Z. Z. et al, Fed. Proc. 28:759, 1969.
 22. Ziporin, Z. Z. et al, Calc. Tiss. Res. 13:187, 1973.

effect on calcium absorption in the rat. This was supported by the findings of Drescher and DeLuca (23,24) which suggested that intestinal mucosa of a D-deficient rat contained a protein which was converted to CaBP when the vitamin was administered.

Thus, the question regarding the role of vitamin D in absorption of calcium by the intestine is unresolved. It is not known whether events triggered by vitamin D require prior protein synthesis or activation of existing proteins. Therefore, it appeared reasonable to investigate distribution of mucosal proteins in D-deficient rats as compared with D-repleted rats and to assess the response of these proteins to vitamin D by measuring changes in the amounts of the proteins and their incorporation of labeled amino acids. As will be seen, the interpretation of data derived from such experiments requires a distinction between net de novo protein synthesis and turnover of existing proteins.

Kidneys from the same animals were treated in a fashion similar to the mucosal tissue. Since kidneys also have CaBP (25) and are the site in which 1α -hydroxylations occur leading to formation of $1,25(\text{OH})_2\text{D}_3$, the active metabolite of vitamin D, we compared the responses of mucosal and kidney proteins to vitamin D.

MATERIALS AND METHODS

<u>BUFFER</u>	<u>LABELLED AMINO ACIDS</u>
0.34 M Sucrose	^{14}C -L-Lysine (UL)
50 mM Tris	^{14}C -L-Glutamic Acid (UL)
20 mM KCl	^{14}C -L-Leucine (UL)
5 mM MgCl_2	
0.20 mM CaCl_2	

Labeled amino acids were obtained from New England Nuclear Corp. at the highest specific activity available. They were combined in distilled-demineralized water and administered as 0.1 ml of solution intubated each day for two days. Thus, animals received 6.25 μCi of each amino acid during the period of treatment. These amino acids were selected because they contribute significantly to the composition of CaBP (18).

-
23. Drescher, D. and DeLuca, H. F., Biochemistry 10:2302, 1971.
 24. Drescher, D. and DeLuca, H. F., Biochemistry 10:2308, 1971.
 25. Corradino, R. A. and Wasserman, R. H., Arch. Biochem. Biophys., 126:957, 1968.

Animals: Twenty Sprague-Dawley weanling rats of either sex were 21 days old and 50-60 grams body weight at the time they were received. Litter mates, regardless of sex, were distributed among the experimental diet groups.

Diets: Diet 1 - 1.2% Ca:0.02%P

Cornstarch 69.20%; egg albumin 15.40%, vitamin mix 0.51%, trace elements 1.03%, alphacel 5.0%; salt mix 4.00%; CaCO_3 1.905%; Wesson oil 30 ml/kg.

Diet 2 - 0.02% Ca:0.3%P

The composition is described in a previous paper, as is the vitamin mix, the trace elements mix and the salt mix (22).

Treatments: The animals were divided into groups and fed diet 1 and diet 2. They were treated for 16 days according to the protocol described in "Experimental Procedures" (22).

On the 16th day, half the animals of each group (5) received 1000 IU vitamin D_3 by mouth while controls received an equal volume of diluent (Wesson oil). The animals continued on their respective diets. Twenty-four hours later, the food cups were removed. Two hours after removing the food cups the animals were intubated with a solution containing the radioactive amino acids in distilled-demineralized water. Two hours after intubation, the food cups were replaced, resuming food and water ad libitum. The next day the program was repeated except for the administration of vitamin D. Approximately 48 hours after the first intubation with amino acids the animals were sacrificed.

At sacrifice, the animals were anesthetized with ether so that blood could be drawn from the abdominal aorta to the point of exsanguination. From each animal, a 5-6 cm strip of duodenum just distal to the pylorus was excised, slit open, and rinsed in ice cold wash solution (22). The strip was blotted dry on filter paper and placed on a clean glass microscope slide kept cold by being placed on ice. The mucosa was scraped by passing the edge of a microscope slide over the intestine. The scraped material was gathered and transferred to a centrifuge tube which also served as an homogenizing tube. The mucosae of the animals in each group were pooled and suspended in an equal volume of ice cold buffer. They were then centrifuged at 3000 rpm for 30 minutes in a refrigerated centrifuge maintained at 4°C , as were all subsequent centrifugations. The supernatants were drawn off and discarded. The remaining cells were resuspended in 1 ml of buffer and homogenized with a teflon pestle by slowly rotating while moving the pestle slowly up and down several times. The homogenate was centrifuged at $600 \times g$ for 10 minutes to sediment nuclear debris and large particles. The supernatant was removed and centrifuged at $5000 \times g$ for 10 minutes to sediment the mitochondria. The supernatant of this spin was centrifuged at $54000 \times g$ for 60 minutes to

sediment microsomes and ribosomes. The supernatant was centrifuged at 105000 x g for two hours to remove all remaining particulate material and provide a supernatant containing soluble proteins.

Pellets from the various centrifugations, as well as the final supernatant, were made up to a known volume with buffer and suspended to disperse the proteins in solution. Aliquots were taken for protein analysis by the method of Lowry (26). At the same time, aliquots were taken for liquid scintillation counting in Brays solution (27).

Gel Electrophoresis: Fifty to 100 μ l aliquots of the soluble proteins, which remained in the supernatant after the 105000 x g spin, containing approximately 200-400 μ g of protein/50 μ l, were subjected to gel electrophoresis on 7% polyacrylamide prepared from cyanogum 41 purchased from the EC Apparatus Corp. The buffer system used was 89 mM Tris, 2.5 mM disodium EDTA, and 88.5 mM boric acid at pH 8.3. The gels were run at 5 ma/tube for 40 minutes in a Shandon Analytical Polyacrylamide Gel Electrophoresis Apparatus. The gels were stained for 60 minutes in 0.2 gm amido black 10B, 50 ml methanol, 50 ml H₂O, 10 ml glacial acetic acid and destained electrophoretically in 7% acetic acid using the same apparatus at 7.5 ma/tube.

Each gel was transferred to a 10 x 75 mm test tube, covered with 7% acetic acid and placed in a Clifford Instruments Densitometer, model 345, for optical density measurement of stained protein bands. The scan length was on the 10 cm scale for mucosa preparations and on the 5 cm scale for the kidney preparations. Following density measurements, the gels were sliced in a Gilson Gel Fractionator providing 1 mm segments. The minced gel segments were collected in scintillation vials and 0.1 ml 30% H₂O₂ added. After overnight digestion at 50°C, 15 ml of liquid scintillation counting solution were added (10% Beckman Bio-Solv solubilizer, BBS-3, in Toluene and Fluors). Separate tests revealed that the amounts of H₂O₂ used did not significantly affect the validity of the pyridine quench correction curves.

Each radioactive amino acid was applied to a gel column and subjected to the same electrophoretic procedure as the protein fractions. There was no radioactivity in the gels at the end of the run. This would establish that free labeled amino acids did not contaminate gel slices taken for subsequent counting.

The effects of varying dietary content of calcium and phosphorus, as well as short-term administration of vitamin D, on protein synthesis in duodenal mucosa and in kidney were assessed. Protein synthesis or protein

26. Lowry, O. H. et al, J. Biol. Chem. 193:265, 1951.

27. Bray, G. A., Anal. Biochem. 1:279, 1960.

turnover was determined in the subcellular fractions and soluble proteins by measuring the amounts of protein and the amounts of ^{14}C -amino acids incorporated per unit weight of protein.

While the tissues taken for the mucosa and kidney preparations were not weighed, an effort was made to make each length of mucosa and each quarter of the kidney approximately equal. Therefore, there is some basis for drawing attention to the apparent effect of vitamin D on the total protein content of these tissues. This was revealed as lesser amounts of total protein in the mucosa of animals treated with the vitamin (Table 1); the kidneys of these animals had somewhat higher amounts of protein after the same treatment.

Interpretation of Labeled Amino Acid Data: The interpretation of data from the soluble proteins in the 4 groups of animals was based upon the following:

- 1) The appearance of new protein peaks with high specific activity was interpreted as de novo synthesis.
- 2) When comparison of identical bands common to two or more groups revealed increased amounts of protein in the band of one group associated with increased incorporation of radioactive amino acids, this was interpreted as increased synthesis of an existing protein.
- 3) If the amount of protein in a particular band was the same for two or more groups, but showed higher specific activity in one group, this was considered to be increased turnover of an existing protein.
- 4) Where two groups had a common band but one had lesser amounts of protein and higher specific activity, it was considered to indicate that the pool of protein was smaller at the time the labeled amino acids were available, and active synthesis or increased turnover was occurring.
- 5) Where there was decreased amount of protein and lower specific activity (Table 1, Group II vs IIa), this was interpreted as either a lower rate of protein synthesis or increased rate of degradation.

RESULTS AND DISCUSSION

¹⁴C-Amino Acid Incorporation Into Subcellular Fractions: The data (Table 1) demonstrate that cellular proteins of the mucosa are found in the soluble, nuclear debris, ribosome-microsome, and mitochondrial fractions, in that order of decreasing quantity. The ribosome-microsome fraction will be referred to as the microsome fraction. In animals fed diet 1, vitamin D increased the proportion of protein in the nuclear debris, microsomal and mitochondrial fractions and decreased the proportion of protein in the soluble protein fraction. Furthermore, vitamin D appeared to increase the specific activity of the soluble proteins, the nuclear debris and microsomal fractions with marginal or no increase in the mitochondrial fraction.

The distribution of proteins in the kidneys (Table 2) was somewhat different from that of the mucosa in three of the four groups. Nuclear debris proteins contributed the largest protein fraction, followed in order by microsomes, soluble protein and mitochondria. The kidneys of animals fed diet 1 with and without vitamin D had a much greater proportion of cellular proteins in the nuclear debris fraction than in the microsomal fraction, while those fed diet 2 had virtually equal proportions. Animals fed diet 1 with vitamin D resulted in an increase in microsomal proteins while decreasing the proportion of nuclear debris protein. This was not observed in animals fed diet 2 although they received the same vitamin D treatment. Specific activities of the various protein fractions in the kidney were lower than those encountered in the mucosal proteins. The kidney microsomal proteins of animals fed diets 1 and 2 had lower specific radioactivities than did the other protein fractions, but this was not interpreted as representing lower synthetic activity since the total amount of microsomal protein represented a significant fraction of the total protein. The low specific radioactivity may have been due to protein synthesis in response to vitamin D occurring prior to availability of labeled amino acids.

Gel Electrophoretic Separation of Mucosal Soluble Proteins and Associated Radioactivity: The protein bands and resultant patterns produced by gel electrophoresis allowed a comparison of the soluble proteins contained in the mucosal extracts. While identical proteins are presumed to have the same rate of migration on gels, we have found what appeared to be identical bands to be slightly displaced even in duplicate tubes in the same run, the displacement being greater with increasing distance from the origin. Starting with the 8th cm on the protein print-out, the bands were shifted slightly to the left (away from the origin).

Following gel electrophoretic separation, soluble proteins in the mucosa of animals fed diet 1 (Fig. 1a) had the same bands from the origin to the 12th cm, regardless of vitamin D status. Animals not treated with vitamin D showed a shoulder at the 13th cm which may be the pre-CaBP suggested by Drescher and DeLuca (23,24). In animals treated with vitamin D (Fig. 1b) the 13 cm shoulder disappeared and a 16th cm band appeared which could be analogous to the chick intestinal CaBP described by

Wasserman and Taylor (17). A comparison of the two patterns is presented in Figure 1c. As far as we could detect by this procedure, and allowing for the shift in bands as described above, the only difference between the patterns from D-deficient and D-treated animals were the pre-CaBP band in the former group and the CaBP band in the latter.

Figures 1d-f represent bands obtained by gel electrophoresis of soluble proteins from the mucosa of animals maintained on diet 2. Figure 1d, showing the bands from animals without vitamin D, appears to present a shoulder at 13.5 cm from the origin and a prominent band at 15 cm. Figure 1e, depicting bands from animals treated with vitamin D, shows no shoulder at 13.5 cm and a prominent band at 15 cm. A comparison of the two patterns is presented in Figure 1f. If we postulate that the 13.5 cm band is the pre-CaBP and that the 15 cm band is CaBP, some explanation is needed for the appearance of CaBP in the non-vitamin D-treated animals represented by Figure 1d. The most obvious explanation could be the presence of even small amounts of vitamin D as a contaminant in the diet. We consider this unlikely since these animals presented the picture of vitamin D-deficient rats as described by Arnaud et al. (28). In those fed diet 2 (a low calcium diet), blood Ca was less than 6.5mg/100ml, (actual range of values was 4.5-5.5mg/100ml); they stopped growing after three weeks on that diet; litter mates maintained on diet 1 (same ingredients as diet 2, differing only in the amounts of Ca and P salts) manifested the classic signs of vitamin D deficiency. Results from other studies which may be pertinent to an explanation of the present data are those reported by Morrissey and Wasserman (29) and Ziporin et al. (22). The former authors, using chicks, found an interaction between calcium and phosphorus content of the diet and the level of calcium absorption by the intestine, so that those fed the lowest calcium intake (0.08%) had a high rate of calcium absorption regardless of phosphorus intake. If a direct relationship between calcium absorption and CaBP content exists (17), this would suggest that animals fed a low calcium diet have a higher uptake of calcium because of the presence of CaBP even in vitamin D-deficient animals. Ziporin et al. (22) found higher uptakes of calcium in intestinal slices of D-deficient rats when fed diet 2 as compared to diet 1. We therefore consider the presence of CaBP in D-deficient low calcium animals an important observation and one that is not likely to be due to contamination of the diet with vitamin D. Also, there is no peak of radioactivity in the region of the CaBP band (Figs 1a, 1b).

Gel Electrophoretic Separation of Kidney Soluble Proteins and Associated Radioactivity: Figures 2a-c represent separations of soluble proteins found in kidneys of animals maintained on diet 1. Figure 2a shows

28. Arnaud, C. et al, J. Clin. Invest. 45:1955, 1966.

29. Morrissey, R. L. and Wasserman, R. H., Am. J. Physiol. 220:1509, 1971.

protein bands from those not treated with vitamin D, while Figure 2b represents the same protein fractions from animals given vitamin D. While we are not able to identify the CaBP fraction in these tracings, the band at 10 cm is related to vitamin D treatment. In both Figures 2c and 2f the 10 cm band in vitamin D-treated animals fed both diets is higher than the band from those animals not treated with the vitamin. This suggests a greater amount of this protein in kidneys of D-treated animals. No radioactivity tracing for Figure 2b is included because the counts were too low to be meaningful.

Figures 2d and 2e present the patterns of soluble kidney proteins taken from animals fed diet 2 without vitamin D treatment (Fig. 2d) and with vitamin D treatment (Fig. 2e). The overlay (Fig. 2f) of the two tracings reveals identical bands which differ only with respect to the concentration of protein in the bands. Only in Figure 2e, representing D-treated animals fed diet 2, is there a small amount of radioactivity in the 10 cm band. Interpretation as to whether this radioactivity represents de novo synthesis or increased turnover requires further experimentation.

Effect of Dietary Calcium and Phosphorus on Soluble Mucosal Proteins: The question of whether prior dietary levels of calcium and phosphorus affect the distribution of soluble mucosal proteins may be examined by comparing tracings of non-vitamin D-treated animals fed the two diets (Figs. 1a, 1d) and the vitamin D-treated animals of these groups (Figs. 1b, 1e). As indicated earlier, those not given vitamin D have a pre-CaBP regardless of diet, but in the same area as CaBP. When animals fed either diet were given vitamin D the pre-CaBP band disappeared and a band appeared in the region presumed to represent CaBP.

The remainder of the electrophoretic patterns reveal no protein bands which could be related either to vitamin D or different calcium and phosphorus intakes. Also, while the peaks of radioactivity do not suggest synthesis of new proteins, they do offer a clue which points to specific bands of high incorporation during the test period when labeled amino acids were administered.

COMMENT

Before attempting to interpret data presented in this paper one must consider the nature of the mucosal material collected. Scraping the mucosa, as described, removes all cellular elements down to the circular muscle of the intestine. Thus, we should expect these preparations to contain: brush border fragments; epithelial cells, some of them goblet cells; elements of the lamina propria; blood vessels and basement membranes. By homogenizing such diverse entities and separating them by centrifugation it is apparent that the mitochondrial preparation (5000 x g) contained particles derived from all cellular elements present in the mucosa. The same heterogeneity should be expected in the nuclear debris and microsomal fractions. And finally, the soluble protein

fraction represents the soluble proteins from all the component cells. Similarly, the kidney preparation must also be viewed as containing proteins from various cellular components whose in vivo functions are significantly different from each other.

The use of specific activity as a measure of the rate of synthesis may lead to erroneous conclusions since we had no information as to the size of the protein pool at the time the labeled amino acids were incorporated. Thus, for example, equal rates of incorporation of labeled amino acids, and presumable equal rates of protein synthesis, could provide widely different specific activities when related to protein pools whose sizes differed at the time synthesis occurred. With this in mind, we see (Tables 1 and 2) that comparable amounts of protein in the soluble and nuclear debris fractions had widely different specific activities for animals fed diet 1 vs diet 2. We cannot conclude that these different values were due solely to different rates of protein synthesis or turnover. Finally, we must consider that amounts of protein measured and their related specific activities were also a function of 1) administration of vitamin D and the initiation of protein synthesis; 2) time elapsed between the administration of vitamin D and initiation of protein synthesis; and 3) the time relationship between the initiation of protein synthesis and administration of labeled amino acids. With these limitations in mind we can report only gross changes in protein content and associated specific activity without designating the cells in which the changes occurred. This must await studies specifically designed for that purpose.

The cellular proteins were divided into two groups; 1) particulate proteins and 2) soluble proteins. In D-deficient animals fed both diets soluble mucosal proteins comprised approximately half the cellular proteins, with the remainder accounted for as nuclear debris (20-30%), mitochondrial (6-10%) and microsomal proteins (11-19%). Following administration of labeled amino acids, the microsomal fraction had the highest specific activity with lesser amounts in the nuclear debris, mitochondrial and soluble proteins. As compared to those fed diet 1, subcellular fractions from animals fed diet 2 resulted in higher specific activities in the nuclear debris and soluble proteins associated with almost equal amounts of protein in these fractions. This indicates a more rapid turnover of these proteins influenced by dietary content of calcium and phosphorus.

Administration of vitamin D produced two main results; 1) a decrease in the total amount of protein in the mucosa when compared to D-deficient animals; and 2) a shift in the ratio of soluble and particulate protein to the total amount of mucosal protein. The soluble protein ratio decreased while the particulate protein ratio increased. Whether these changes are related or occur independently of each other is not known.

Our findings provide a basis for speculating on the possible course of events occurring in the duodenal mucosa as they relate to the effects

of vitamin D, the dietary intake of calcium and phosphorus, and synthesis of cellular proteins both particulate and soluble.

It has been reported (30) that 8 to 15 hours are required for the intestine to respond to vitamin D by increased absorption of calcium. While little is known of the biochemical events taking place during this time lag, metabolic changes have been reported which may be related to the vitamin and enhanced calcium absorption. Stohs et al. (12) reported an increased incorporation of ^3H -orotic acid in RNA of mucosal cells within three hours following treatment with vitamin D. Birge and Alpers (14) found that vitamin D enhanced DNA synthesis in intestinal mucosa within four hours, and that this preceded stimulation of calcium transport by the intestine. They related the enhanced DNA synthesis and increased calcium absorption to increased numbers of cells per cm of intestine, or net cell synthesis. LeBlond and Stevens (31) reported normal turnover time of rat epithelial cells from the duodenum to be 1.57 days. We would assume "normal" to mean absence of pathologic lesions as well as sufficient amount of vitamin D in an otherwise adequate diet.

In the early stages following administration of vitamin D to a D-deficient rat (3-4 hours), increased synthesis of RNA and DNA, as reported by Stohs et al. (12) and Birge and Alpers (14), would support the view that there is a net increase in premitotic and mitotic activity with a likely increase in cell population of the mucosa. The relation between these events and enhanced absorption of calcium at 8-15 hours after dosage with vitamin D is not known. The finding that vitamin D-treated rats on both diets have less total protein than their non-treated counterparts could suggest an increased sloughing of cells associated with a decreased maturation time in rats of the former group. The newly-formed cells would appear to have less protein, but would concentrate available protein in the particulate fractions, especially the nuclear debris, rather than the soluble protein fraction. A comparison of the protein distributions in the mucosa of D-treated rats on diets 1 and 2 reveals similar amounts of protein in the particulate and soluble protein fractions. However, the finding that the nuclear debris and soluble protein fractions of those fed diet 1 have a higher specific activity than those fed diet 2 would suggest that protein synthesis occurred in rats on diet 1 when labeled amino acids were available, while synthesis occurred in those fed diet 2 before labeled amino acids were present.

The bands separated by gel electrophoresis and their associated radioactivity revealed some interesting effects of dietary content of calcium and phosphorus as well as vitamin D. As reported by Drescher and DeLuca (23, 24) we found a band similar to the one designated as

30. DeLuca, H. F., Fed. Proc. 28:1678, 1969.

31. LeBlond, C. P. and Stevens, C. E., Anat. Rec. 100:357, 1948.

pre-CaBP in animals deficient in vitamin D. This band disappeared following treatment with the vitamin. CaBP was found in all animals given vitamin D and was also found in D-deficient animals fed diet 2. If the band labeled by Drescher and DeLuca (23, 24) is in fact a pre-CaBP which is altered to become CaBP when vitamin D is administered, then conversion of pre-CaBP to CaBP would not appear to require de novo protein synthesis in response to vitamin D, and actinomycin D would not inhibit this conversion. The failure of actinomycin D to inhibit vitamin D has been reported by Ziporin et al. (21, 22). Our finding that there is little or no radioactivity incorporated in the region of the CaBP would support the above conclusion that in the rat there is no de novo protein synthesis in conversion of pre-CaBP to CaBP.

As for the kidney, neither dietary levels of calcium and phosphorus nor the presence or absence of vitamin D significantly affect the distribution of proteins in the particulate or soluble fractions. Incorporation of labeled amino acids into specific renal proteins was not affected by either vitamin D or diet. While there is no suggestion of a pre-CaBP in kidney tissue from rats with any of the treatments, CaBP appears in kidney preparations of D-deficient rats fed both diets and vitamin D increases the amount of CaBP in rats fed both diets.

CONCLUSIONS AND RECOMMENDATIONS

These results point to the need for further investigation. The influence of vitamin D on the rate of maturation and turnover time of mucosal epithelial cells could be a significant factor in understanding mucosal physiology. The effect of dietary levels of calcium and phosphorus on protein synthesis in this tissue may be a factor in understanding the differences in calcium absorption of animals on high or low calcium intakes. With the present data at hand more definitive experiments may be designed.

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Figure 1. Gel electrophoretic patterns and associated radioactivity of soluble proteins from duodenal mucosa

Figure 2. Gel electrophoretic patterns and associated radioactivity of soluble proteins from rat kidney

A P P E N D I X A

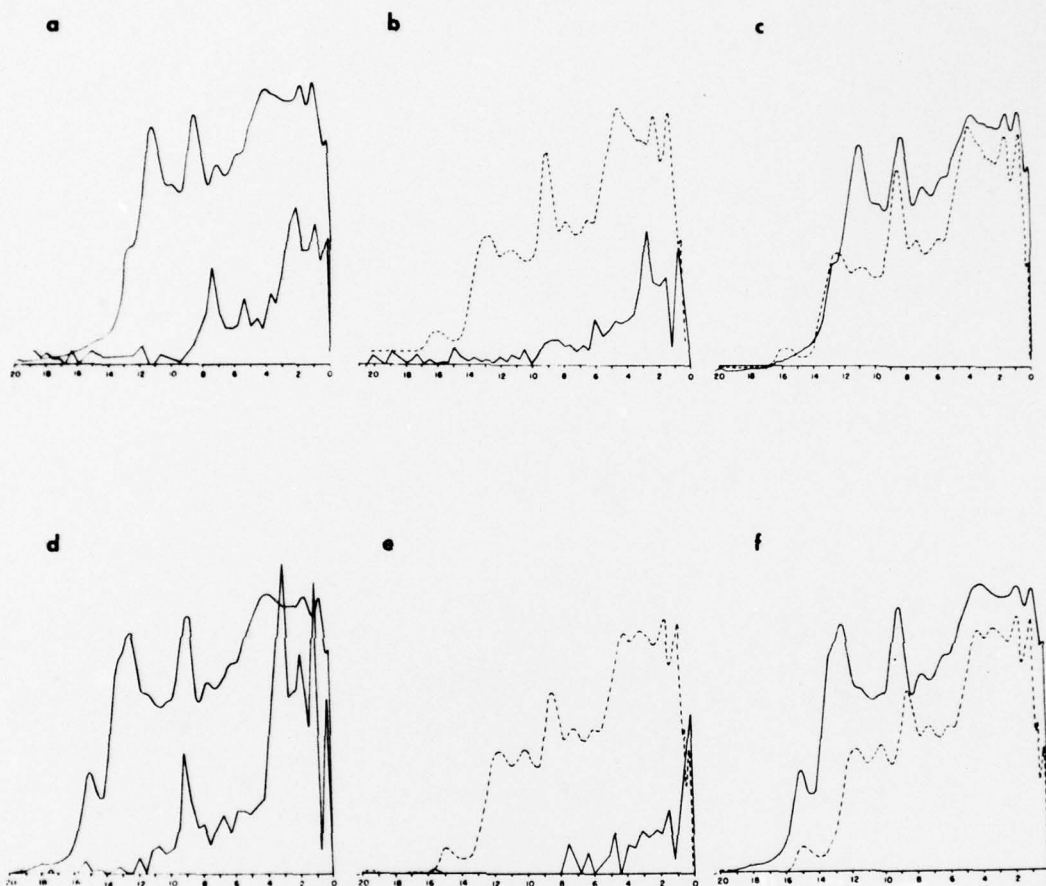


Figure 1. Gel electrophoretic patterns and associated radioactivity of soluble proteins from duodenal mucosa. a) Electrophoretic pattern (upper line) and associated radioactivity (lower line) for D-deficient rats fed diet 1, b) electrophoretic pattern (broken line) and associated radioactivity (solid line) for D-treated rats fed diet 1, c) electrophoretic pattern comparison of a and b, d) electrophoretic pattern (upper line) and associated radioactivity (lower line) for D-deficient rats fed diet 2, e) electrophoretic pattern (broken line) and associated radioactivity (solid line) for D-treated rats fed diet 2, f) electrophoretic pattern comparison of d and e.

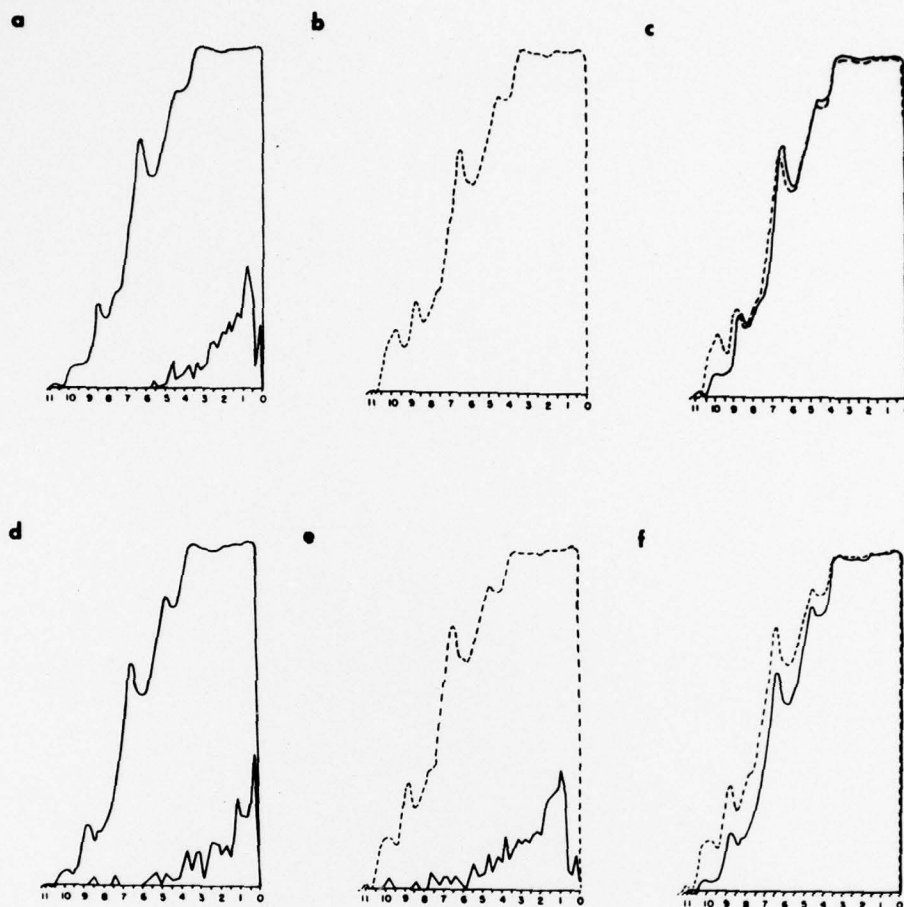


Figure 2. Gel electrophoretic patterns and associated radioactivity of soluble proteins from kidney. a) Electrophoretic pattern (upper line) and associated radioactivity (lower line) for D-deficient rats fed diet 1, b) electrophoretic pattern (broken line) for D-treated rats fed diet 1 (radioactivity count rates were too low to be meaningful), c) electrophoretic pattern comparison of a and b, d) electrophoretic pattern (upper line) and associated radioactivity (lower line) for D-deficient rats fed diet 2, e) electrophoretic pattern (broken line) and associated radioactivity (solid line) for D-treated rats fed diet 2, f) electrophoretic pattern comparison of d and e.

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Table 1. The effect of vitamin D₃ and dietary intake of calcium and phosphorus on the distribution and specific activity of proteins from rat duodenal mucosa

Table 2. The effect of vitamin D₃ and dietary intake of calcium and phosphorus on the distribution and specific activity of proteins from rat kidney

APPENDIX B

TABLE 1

The effect of vitamin D₃ and dietary intake of calcium and phosphorus on the distribution and specific activity of proteins from rat duodenal mucosa

	Group I Mucosa			Group Ia Mucosa		
	μg fraction	% of total protein	dpm/ μg prot.	μg fraction	% of total protein	dpm/ μg prot.
Nuclear Debris (600 x g)	16840	29	7.62	9200	32	10.37
Mitochondria (5000 x g)	3588	6	5.23	2838	10	6.78
Microsome (54000 x g)	6440	11	8.91	5510	19	13.30
Pellet (105000 x g)	939	1	2.94	733	2	3.30
Soluble Protein	30840	53	4.12	10530	37	10.57
Total Protein (μg)	58647					
	Group II Mucosa			Group IIa Mucosa		
	μg fraction	% of total protein	dpm/ μg prot.	μg fraction	% of total protein	dpm/ μg prot.
Nuclear Debris (600 x g)	10488	21	9.38	7850	27	7.87
Mitochondria (5000 x g)	4725	10	6.92	2633	9	7.75
Microsome (54000 x g)	7370	15	10.15	4910	17	11.45
Pellet (105000 x g)	922	2	3.64	737	2	3.25
Soluble Protein	25680	52	9.89	13440	45	5.47
Total Protein (μg)	49185			29570		

Group I - 1.2% Ca: 0.02% P diet, no vitamin D₃
 Group Ia - Same diet as Group I, treated with 1000 IU vitamin D₃ three days before sacrifice
 Group II - 0.02% Ca: 0.3% P diet, no vitamin D₃
 Group IIa - Same diet as Group II, vitamin D₃ treatment same as Group Ia

TABLE 2

The effect of vitamin D₃ and dietary intake of calcium and phosphorus on the distribution and specific activity of proteins from rat kidney

	Group I Kidney			Group Ia Kidney		
	μg protein fraction	% of total protein	dpm μg protein	μg protein fraction	% of total protein	dpm μg protein
Nuclear Debris (600 x g)	27970	44	2.28	26580	37	2.53
Mitochondria (5000 x g)	7884	12	1.82	6804	10	2.08
Microsome (54000 x g)	8540	13	1.80	16904	24	0.79
Pellet (105000 x g)	7675	12	1.80	7501	11	2.15
Soluble Protein	12000	19	1.80	12120	17	1.78
Total Protein (μg)	64069			69909		
	Group II Kidney			Group IIa Kidney		
	μg protein fraction	% of total protein	dpm μg protein	μg protein fraction	% of total protein	dpm μg protein
Nuclear Debris (600 x g)	24140	30	1.99	30060	33	2.12
Mitochondria (5000 x g)	8093	10	1.69	8302	9	1.63
Microsome (54000 x g)	27496	35	0.61	30144	33	0.65
Pellet (105000 x g)	8302	10	1.76	9348	10	1.64
Soluble Protein	11550	15	1.43	13830	15	1.22
Total Protein (μg)	79581			91684		

Group I - 1.2% Ca: 0.02% P diet, no vitamin D₃
 Group Ia - Same diet as Group I, treated with 1000 IU vitamin D₃ three days before sacrifice
 Group II - 0.02% Ca: 0.3% P diet, no vitamin D₃
 Group IIa - Same diet as Group II, vitamin D₃ treatment same as Group Ia

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